2013

The Hormonal and Genetic Regulation of Melanization and Phenotypic Plasticity in the Milkweed Bug, Oncopeltus fasciatus

Tiffany Chen
tchen2@wellesley.edu

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Tiffany Chen

Advisor: Dr. Yuichiro Suzuki

Submitted in Partial Fulfillment of the Prerequisite for Honors in the Department of Biological Sciences

April, 2013

Acknowledgements

I would like to thank my thesis and major advisor Professor Yuichiro Suzuki for all his guidance and support throughout my college career and for things beyond. It was through his help that I was able to have my first summer research experience, which opened so many doors to exploring research fields beyond what are available at Wellesley. You are the best mentor ever!

I would also like to thank Professor David Ellerby and Professor Christa Skow for their help, guidance, and encouragement through this project.

Thank you to the Office of the Dean of Wellesley College and the National Science Foundation for their financial support of my thesis project.

Thanks to all current and past members of the Suzuki lab, who have made my college research experiences at Wellesley so fun and amazing. And special thanks to Leila Chaieb for helping me with a part of this project.

I would like to thank my family for being there for me, if not in person then in spirit, and supporting me in all my major life decisions.

Finally, I would like to thank my friends who provided me with support through all of my college experiences and hilarious anecdotes that I will share with people for the rest of my life. I appreciate that they understand and enjoy my sense of humor.

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ABSTRACT

Insect pigmentation provides an excellent system for understanding the hormonal and genetic processes underlying phenotypic plasticity. Phenotypic plasticity describes the ability of the same genotype to produce different phenotypes in response to the environment. When a discrete switch exists in the phenotypes produced in response to a continuously varying environmental input, the plastic trait is known as a polyphenism. Developmental hormones are known to control polyphenisms, but the origins of polyphenisms remain poorly understood. Using double-stranded RNA injections, the expression of hormone receptor genes and a Hox gene was knocked down to explore the genetic and hormonal mechanisms underlying abdominal pigmentation regulation and plasticity in the milkweed bug, *Oncopeltus fasciatus*. Studying how plasticity in melanization is regulated has implications for our understanding of the origins of polyphenisms.

INTRODUCTION

1. Overview of Phenotypic Plasticity

All traits expressed by an organism are dictated not only by its genotype, but also by environmental influences (Nijhout, 2003). Phenotypic plasticity is the ability of a single genotype to express a range of phenotypes in response to different environmental stimuli (Debat and David, 2001; West-Eberhard, 2008). Plastic traits can be characterized through reaction norms that graphically describe the range of environmental variation that is translated into a spectrum of phenotypic variation (Suzuki et al., 1986; Via et al., 1995; Nijhout, 2003; West-Eberhard, 2008). An example of a reaction norm is the increased relative root biomass and leaf area of plants in response to subpar soil nutrient and light conditions (Sultan, 2000). A subset of phenotypically plastic traits expresses a discontinuous spectrum of phenotypes with a discreet switch in the phenotype along a continuous environmental input. These traits are known as polyphenisms, and are mostly regulated by developmental hormones. Some examples of polyphenisms in insects include: horn growth in male dung beetles and the two different seasonal forms of the butterfly wing coloration. Male dung beetles, *Onthophagus taurus*, that achieve a threshold size develop long horns, while their smaller counterparts develop either rudimentary horns or none at all (Emlen and Nijhout, 2001). The buckeye butterfly's adult form is dictated by temperature and photoperiod cues, with the wing pigmentation of the summer and winter forms differing in coloration (Rountree and Nijhout, 1995).

In the past, plastic phenotypes in studies were ignored as experimental inconsistencies or seen as unfortunate failures of the genetic apparatus (Falconer, 1952; Wigglesworth, 1961; West-Eberhard. 1989). Since then, more attention has been given to

the influence of the environment that can result in dramatic differences in the expressed phenotype in organisms with similar genotypes. It is now evident that virtually any trait can be phenotypically plastic to a quantifiable degree. While morphological traits are most obvious to a casual observer (Schlichting and Pigliucci, 1998), even physiological and behavioral traits are phenotypically plastic and influenced by the environment (Uvarov, 1921; Agrawal, 2001; Schlichting and Smith, 2002; West-Eberhard, 2008; Whitman and Agrawal, 2009). Phenotypic plasticity can generate spatial and temporal variation in development, with the effects persisting for generations (Agrawal et al., 1999; Fordyce, 2006). Highly plastic responses, such as animal behavior, can happen immediately in response to interactions with other organisms, and are easily reversible, while seasonal polyphenisms expressed in adult butterflies of some species are in response to environments experienced before the penultimate molt and permanent (Nijhout, 2003; West-Eberhard, 2003).

The evolution of phenotypic plasticity

As it is impossible to decouple the influences of the external and internal environment from phenotypic expression, all traits are plastic to some degree (Schlichting and Smith, 2002; Nijhout, 2003). Thus, the ability to respond to novel environmental changes through phenotypic plasticity is not inherently adaptive as it is merely a response to the environment. However, some traits are more plastic than others. As plasticity came to be regarded as something more than just developmental noise, the question raised by biologists became whether plasticity was a trait that could be acted on by evolutionary forces.

Detractors such as Via (1993) denied that selection for plasticity occurred in nature. She claimed natural selection is always stabilizing or directional, and that adaptive phenotypic plasticity for ecologically important characteristics such as morphology and physiology are a by-product of selection toward different phenotypic values within a variable environment. There has, however, arisen a general agreement in the field that adaptive plasticity, such as polyphenism, can evolve under natural selection (Stearns, 1989; de Jong, 2005). There is also the acknowledgement that natural selection leads to the loss of plasticity if a certain expressed form of a trait results in higher fitness than if the other were expressed (Schlichting and Smith, 2002).

The more recent consensus in the field is that plasticity is a quantitative trait that is acted on by natural selection, as has been found in experiments where the plasticity of a trait has been selected for and against (Scheiner, 1993; Suzuki and Nijhout, 2006). For example, through their studies on the temperature-dependent plasticity of thoracic size in *Drosophila melanogaster*, Scheiner and Lyman (1991) demonstrated that the temperature-regulated plasticity of thorax size responded to selection. They found that the flies were able to reach a selection limit of zero plasticity in the lines selected for decreased plasticity (Scheiner and Lyman, 1991). For the lines selected for the increased plasticity, the authors found that there was the development of increased response over time, but the change in response was not as dramatic as in the decreased plasticity line.

Polyphenism is another example of how phenotypic plasticity can evolve under natural selection. One example is the polyphenic hornworm caterpillar coloration response to temperature differences. In the tomato hornworm, *Manduca quinquemaculata*, a sister species to the tobacco hornworm, *Manduca sexta*, caterpillars are black when reared in

temperatures below 20°C and green when raised in temperatures above that (Hudson, 1966). Suzuki and Nijhout (2006) used mutant black *M. sexta* larvae to demonstrate the flexibility of gaining a polyphenic response. When heat shocked, the caterpillars express a range of color phenotypes from black to green in response. They bred a monophenic line and a polyphenic line, by choosing and breeding the darkest and greenest caterpillars respectively after treatment. After the seventh generation, the monophenic line stopped producing color change despite heat shock treatment, demonstrating the rapid loss of the plastic response (Suzuki and Nijhout, 2006). By the thirteenth generation, the polyphenic line was producing nearly perfectly green caterpillars (Suzuki and Nijhout, 2006). The monophenic line and polyphenic line are examples of genetic assimilation and accommodation respectively. This gain and loss of polyphenic response over a relatively short generational timeline suggests that the polyphenisms can be gained and lost quickly once the mechanisms for regulating it have been established.

The evolution of plasticity is limited by developmental constraint and the inherent cost of maintaining the ability to respond phenotypically to environmental variation (Moran, 1992; DeWitt et al., 1998). Developmental constraint limits the development of plasticity to a certain extent as the morphology and physiology of an animal cannot change too drastically even in response to a novel environment (Debat and David, 2001). Observations of the rapidity of polyphenism evolution, however, suggest that it is possibly the availability of environmental variation that limits the evolution of plasticity rather than the flexibility of developmental systems (Moran, 1992; Schlichting and Smith, 2002). The costs of maintaining plasticity is generally defined as a decrease of fitness due to the organism's ability to express a plastic phenotype even when the optimum phenotype is

generated by the plastic response (Relyea, 2002; Pigliucci, 2005; Auld et al., 2010). Examples of such costs include maintenance costs from the energy required to maintain the sensory and response mechanisms for detecting and responding to environmental changes, and production costs that individuals incur over expressing a plastic response phenotype compared to canalized individuals expressing the same phenotype (Dewitt et al., 1998). That phenotypic plasticity persists despite the inherent costs suggests that the ability to produce a plastic response is overall advantageous or at least not detrimental.

There is great interest in understanding the development and evolution of the genetic mechanisms that confer the ability to be sensitive to environmental cues and respond by expressing different phenotypes (Nijhout, 1999; Agrawal, 2001; West-Eberhard, 2003; Pigliucci, 2005). The genotype's capacity to react to environmental stimuli determines the strength of the resulting phenotypic response (Stearns, 1989; Agrawal, 2001). It is this aspect of phenotypic plasticity that is acted upon by natural selection as any other quantitative trait (Stearns, 1989; Via et al., 1995). However, little is known about the developmental mechanisms that produce a particular reaction norm and how those developmental mechanisms change when reaction norms evolve (Nijhout, 1999).

Phenotypic plasticity and its role in evolution

In the last few decades, there have been debates over the role of phenotypic plasticity in evolution. Some argue that phenotypic plasticity mainly buffers the effects of natural selection (Gillespie and Turelli, 1989; de Jong, 2005). Others argue that plasticity promotes the evolution of novel phenotypes, divergence amongst populations, the formation of new species and adaptive radiation (Moran, 1992; West-Eberhard, 2005;

Pfennig et al., 2010). Both outcomes are possible, depending on the circumstances under which selection is taking place and the trait being selected for (Whitman and Agrawal, 2009; Wund, 2012). There are a number of key questions about the role of plasticity in promoting evolution that need to be answered to convince detractors (Wund, 2012). This process is made difficult because plasticity acts within the lifetime of individuals, while evolution is a generational process and that plasticity itself is acted on by evolution (Wund, 2012).

Phenotypic plastic traits can buffer the effects of natural selection by maintaining genetic variation within a population (Gillespie and Turelli, 1989). Some question if there is enough empirical evidence to support the idea that plasticity has a role in promoting evolution (de Jong, 2005). de Jong (2005) maintains that while current research supports the idea that phenotypic plasticity is a quantitative trait similar to any trait that can be acted on by natural selection, there is no empirical or model support that plasticity promotes evolution. She believes that the idea of natural selection being able to act on shaping plasticity is mutually exclusive from the ability of phenotypic plasticity to facilitate evolution (de Jong, 2005).

Contrary to the claims of de Jong (2005), there are studies that show that phenotypic plasticity plays a role in promoting the development of novel phenotypes and the diversification within populations and species that live in different environments (West-Eberhard, 2005; Pfennig et al., 2010). Changes in the environment can release cryptic genetic variation through phenotypic plasticity, impacting the rate of evolution (McGuinan et al., 2011). If the novel environment persists, the form and expression of newly adaptive traits will continue to be shaped by natural selection and may become

genetically fixed (Scoville and Pfrender, 2010). One example is the ability of the tobacco hornworm caterpillars to produce a coloration response to temperature differences, and for that coloration response to become fixed through both genetic assimilation and genetic accommodation (Suzuki and Nijhout, 2006). Faster evolution of diverse populations can occur in situations of disruptive selection that favors divergent phenotypes, where natural selection acts on the underlying variation of the developmental genetic mechanisms within the population that generate the different phenotypes (Martin and Pfennig, 2010).

These debates highlight the need for an understanding of the developmental control of phenotypic plasticity. Understanding the mechanisms underlying plasticity and how they evolve will be critical to understand the role of phenotypic plasticity in evolution. Given the advances made in molecular biology, this is now possible.

Hormone regulation of polyphenisms

Much is known about the hormonal players that mediate environmental influences on polyphenisms. In insects, most, if not all, polyphenic traits are likely regulated by endocrine control systems that are coupled to environmental cues (Nijhout, 1994; Nijhout, 1999; Riddiford et al., 2000; Nijhout, 2003). Dark color-inducing neurohormone induces the black pigmentation seen in the gregarious form of the locust (Tanaka and Pener, 1994). Usually these systems are regulated by developmental hormones ecdysone and juvenile hormone (JH) (Nijhout, 1999; Nijhout, 2003). Ecdysone is secreted by the prothoracic gland and converted to its common form 20-hydroecdysone (20E) while JH is produced by the corpora allata of most insects (Riddiford, 2012). JH plays a role in regulating the green pigment seen in the solitary form of the locust, *Locusta migratoria* (Tanaka, 2000). In *Precis*

coenia, the buckeye butterfly with the two seasonal forms, the levels of 20E begin to increase at 20 hours after pupation during the long day conditions while 20E levels do not begin increasing in the short day conditions until the ecdysteroid-sensitive period is over (Rountree and Nijhout, 1995).

Hormone control of polyphenisms relies on hormone concentration and timing of hormone secretion, and also the sensitivity of the receptors to the hormone signal (Nijhout, 1999). The removal of the *P. coenia* brain less than 28 hours after pupation results in the development of the short day adult phenotype in more than 50% of individuals, even under long day conditions (Rountree and Nijhout, 1995). The long day adult phenotype can be rescued as long as 20E is injected within 36 hours of pupation. Polyphenisms can evolve from either of the two controls of signaling, hormonal secretion and response, becoming sensitive to the environment (Nijhout, 1999). Once the hormonal control mechanisms of a polyphenism evolves, the ability for a polyphenic response can be gained and lost in a relatively rapid manner as seen in the experiments carried out by Suzuki and Nijhout (2006) in *M. sexta*. Whether the evolution of environmentally sensitive hormonal control of phenotypic plasticity precedes the evolution of a polyphenism, or phenotypic plasticity precedes the evolution of environmentally sensitive hormonal control unique to polyphenisms remains unknown. Studies on the regulation of phenotypic plasticity that has not yet been shaped into a polyphenism are needed to address this issue. Thus, the goal of this study is to begin to address the question of the origin of the hormonal control of polyphenic traits.

2. Insect Pigmentation

The great diversity in insect pigmentation makes it a popular focus for studies in ecology, development, genetics, physiology and behavior (Koch et al., 2000; Wittkopp and Beldade, 2009). Pigmentation is highly variable in terms of colors as well as patterning and location, and is used for inter- and intra-species visual communication, and physiological processes, such as desiccation resistance and thermoregulation (Wiernasz, 1989; Wiernasz, 1995; True, 2003; Parkash et al., 2009; Wittkopp and Beldade, 2009). For example, on the butterfly *Pieris occidentalis*, both males and females exhibit sexual choice based on wing patterning that is temperature-sensitive (Wiernasz, 1989; Wiernasz, 1995). Parkash et al. (2009) found that there was a correlation between the percent melanization and desiccation resistance in populations of *Drosophila melanogaster* collected from different portions of India. In six different altitudinal populations of *D. melanogaster*, it was found that the higher altitude populations were melanized to a greater percentage than lower altitude populations even when all insects were raised at a uniform temperature and this correlated to higher levels of cold resistance (Parkash et al., 2010). The highly plastic nature of pigmentation makes it a wonderful system for studying how the genetic and hormone control mechanisms controlling phenotypic plasticity in melanization evolved.

Melanin is a predominant class of insect pigments (Wittkopp et al., 2009). The melanization pathway is well understood, and thus is a good system to study for understanding how the environment influences the mechanisms behind the plasticity of insect pigmentation (True, 2003). The melanization synthesis pathway and its regulatory mechanisms have been extensively studied in model organisms, such as the tobacco hornworm, *Manduca sexta*, and the fruitfly, *Drosophila melanogaster* (Wittkopp et al., 2003; Gibert et al., 2007; Hiruma and Riddiford, 2009). There are two components of the insect

pigmentation process: 1) the biochemical synthesis of the pigments, and 2) positioning of the pigments in space and time (Wittkopp and Beldade, 2009).

Biochemical synthesis pathway of melanization

The major steps of the melanin synthesis pathways in *M. sexta*, *D. melanogaster* and most other insects are similar. Figure 1 gives an overview of the biochemical synthesis pathway of melanin. Phenylalanine is converted to tyrosine and then dopa at the beginning of the branched biochemical pathway that leads to related dopa molecules, which are ultimately converted to black, brown, and yellow pigments (Wittkopp et al., 2009). The conversion of tyrosine to dopa requires the presence of tyrosine hydroxylase (TH). The addition of 3-iodo-tyrosine (3IT), a competitive inhibitor of TH, results in the loss of larval cuticular melanization which can be rescued by dopa *in vitro* (Wright, 1987; Futahashi and Fujiwara, 2005, 2007). Dopa can then either be oxidized by di-phenoloxidase (PO) to eventually form dopa melanin or first converted to dopamine by Dopa decarboxylase (DDC) before oxidation by PO to eventually form dopamine melanin products (Hiruma et al., 1985; Hopkins and Kramer, 1992; Andersen, 2005). PO and DDC are ubiquitous in insects as they are used in cuticular sclerotization (Hopkins and Kramer, 1992; Andersen, 2005).

Hormone regulation of the melanin synthesis pathway

The ecdysteroid signaling pathway, which includes ecdysone and its most common active form, 20E, has been shown to regulate general growth and development as well as the melanin synthesis pathway in both hemimetabolous and holometabolous insects,

insects that undergo incomplete and complete metamorphosis, respectively (Nijhout, 1994; Riddiford et al., 2000; Hiruma and Riddiford, 2009; Wu et al., 2012). In the melanization pathway, the synthesis of dopa decarboxylase (DDC) is controlled by ecdysone and JH (Hiruma and Riddiford, 1985; Hiruma et al., 1985). It has been found that the decrease of 20E levels increases the expression of DDC and TH, which is necessary for insect cuticular melanization (Andersen, 2005; Futahashi and Fujiwara, 2007; Hiruma and Riddiford, 2009). In *D. melanogaster*, ecdysone directly stimulates DDC expression as the removal or mutation of the ecdysone response element results in an 85% reduction of its expression (Chen et al., 2002). JH, along with melanization and reddish coloration hormone (MRCH), is required for the synthesis of a visual pigment, ommochrome, in the cabbage armyworm, *Mamestra brassicae* (Hiruma et al., 1984). E75, an ecdysteroid-induced transcription factor, has two isoforms, E75A and E75B (Zhou et al., 1998). In *D. melanogaster E75A*, a nuclear receptor gene, is shown to be activated as a response to JH level, suggesting that JH might play a role in influencing the ecdysteroid stimulation of melanization (Dubrovsky et al., 2011).

Ecdysone receptor (EcR)

Ecdysone receptor (EcR) has been shown to play roles in the regulation of molting, metamorphosis, and other aspects of insect development (Brown and Truman, 2009). It is activated by ecdysteroids, such as 20E (Nijhout, 1994). Ecdysone signaling regulates the expression of DDC in insect epidermis (Davis et al., 2007). Thus, EcR likely plays a role in insect melanization. Knockdown studies of ecdysone receptor in rice planthoppers by Wu et al. (2012) support that the receptor EcR has a role in regulating melanization as rice

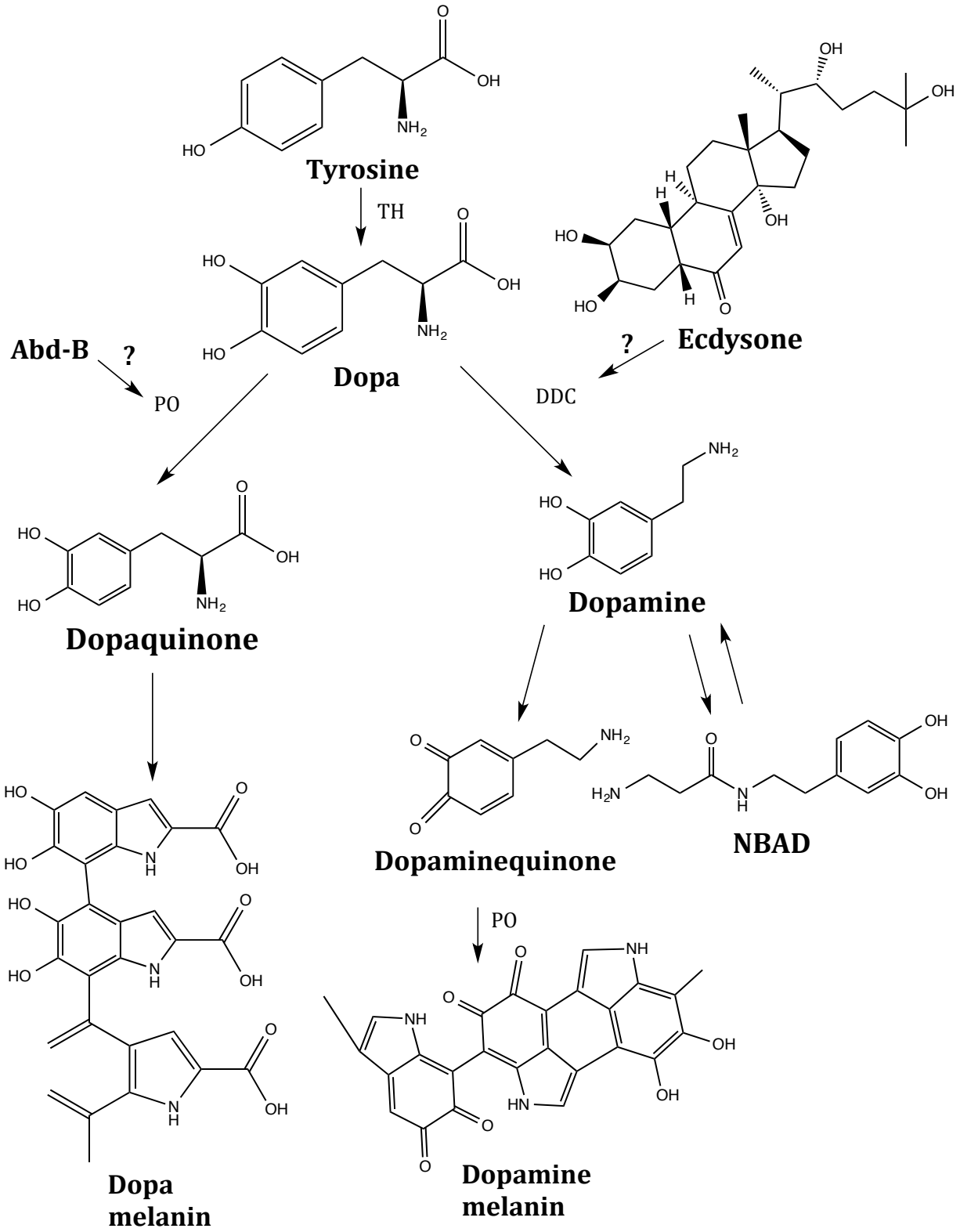


Figure 1. Pathway for the cuticular melanization in insects (after Hiruma and Riddiford, 2009; Wittkopp and Beldade, 2009; Wittkopp et al., 2003). Molecules drawn using ChemBioDraw 13.0.

planthopper wings in EcR knockdown animals are unable to undergo normal melanization after molting.

Fushi Tarazu-factor 1 (FTZ-F1)

Fushi Tarazu-factor 1 (FTZ-F1) is an orphan nuclear receptor that acts downstream of EcR (Hiruma and Riddiford, 2001). The role of FTZ-F1 in the 20E signaling pathway in insect development has been studied extensively in the more derived holometabolous insect, *D. melanogaster* and moderately in the hemimetabolous insect *Blatella germanica*, the German cockroach (Cruz et al., 2008; Ruaud et al., 2010). In *M. sexta*, FTZ-F1 is potentially an activator for DDC expression as both its regulation by 20E is similar to that of DDC and DDC contains a FTZ-F1 response element (Hiruma and Riddiford, 2001; Hiruma and Riddiford, 2009). Dubrovsky et al. (2011) found that in *D. melanogaster*, the removal of FTZ-F1 prevents JH activation of *E75A* expression. In this context, FTZ-F1 knockdown might affect the ecdysone-induced abdominal melanization observed in milkweed bugs.

Methoprene-tolerant (Met)

Methoprene-tolerant (Met) is the receptor of JH. Loss of function studies in the flour beetle, *Tribolium castaneum*, showed that the lack of Met expression confers resistance to JH (Konopova and Jindra, 2007). JH is involved in the melanin synthesis pathway in some insects. The presence of JH inhibits melanin deposition in new *M. sexta* cuticle (Truman et al., 1973; Hiruma and Riddiford, 2009). The absence of JH around the time of head capsule slippage of *M. sexta* leads to the deposition of premelanin granules in the newly forming cuticle (Curtis et al., 1984). In *M. sexta*, the absence of JH leads to a two-fold increase in DDC

activity compared to when JH is present (Hiruma et al., 1985). Thus, JH could potentially act as an inhibitor of pigment deposition.

Pigment positioning

Once melanin has been synthesized, the positioning of the melanin granules is deposited either in random dispersion or in definitive granules, with the degree of melanization dependent upon the number of melanin granules present (Kayser-Wegmann, 1976; Hiruma et al., 1985). One visually dramatic example is formation of butterfly eyespot color rings, which has been shown to result from activation of various transcription factors (e.g. *engrailed, spalt* and *Distal-less*) in the developing wing tissues (Brunetti et al., 2001). In *D. melanogaster*, sex determination genes, signaling pathway genes and Hox genes have been found to play a role in controlling pigment patterning (Kopp et al., 2000; Gibert et al., 2007).

Hox gene: Abdominal-B (Abd-B)

Hox genes are a group of developmental genes that determine segment identity within insects and other vertebrates (McGinnis and Krumlauf, 1992; Cook et al., 2001). They are differentially expressed along the anterior/posterior axis and define segment organization (Akam, 2000). Hox genes are also involved in the complex control of pigmentation pathways in *D. melanogaster* (Kopp et al., 2000; Wittkopp et al., 2003). The *D. melanogaster* homeotic complex is split into the bithorax complex (BX-C) and the Antennapedia complex (ANT-C) (Lewis, 1978).

Abdominal-B (Abd-B) is a posterior Hox gene that is part of the BX-C (Lawrence and Morata, 1991). It has been previously shown to play a role in specifying the identities of

abdominal segments in *Drosophila melanogaster* (Aspiras at al., 2011). Other than functioning in control of body patterning in *D. melanogaster*, Abd-B has been found to regulate pigmentation gene expression in the posterior segments by repressing pigmentation enzymes (Jeong et al., 2006; Gibert et al., 2007; Wittkopp and Beldade 2009). In male fruit flies, transcriptional regulator *bric-a-brac* (*bab*) is repressed by Abd-B to give the male pigmentation pattern (Kopp et al., 2000; Wittkopp et al., 2003). Gibert et al. (2007) also showed that *Abd-B* plays a role in mediating the temperature-mediated plasticity in the abdominal pigmentation of the flies. In the *O. fasciatus* embryo, Hox gene Abd-B plays a role in abdominal patterning and pigmentation repression (Angelini et al., 2005). Thus, *Abd-B* plays a critical role in determining the spatial expression of pigmentation in the *O. fasciatus*. *Abd-B* RNAi in *O. fasciatus* embryos results in ectopic pigmentation on the posterior region of the A7 segment (Angelini et al., 2005). During adult development, *Abd-B* RNAi in the fifth instar nymphs result in overall increased pigmentation such as thicker bands and larger spots, as well as extra ventral melanic pigmentation in segment A6 (A. Sharma, unpublished).

Oncopeltus fasciatus

In this study, *Oncopeltus fasciatus*, the milkweed bug, was used to analyze the mechanism underlying abdominal pigmentation. *O. fasciatus* is a hemimetabolous hemipteran, or true bug, from the family Lygaeidae (Wheeler et al., 2001). It is a good model system for studying the effects of the mechanisms of pigmentation plasticity (Novak, 1955?). *O. fasciatus* abdominal melanic pigmentation is affected by temperature conditions during the sensitive period in the fifth instar stage (Novak, 1951; A. Sharma, unpublished). Its abdominal spots are smaller or absent when the insects are raised at high temperatures,

while these spots are larger when the insects are raised at low temperatures during the sensitive period (A. Sharma, unpublished). This is a trend similar to that seen in different altitudinal populations of *D. melanogaster*; the populations at the higher altitudes, and thus colder temperatures, are darker (Parkash et al., 2010). To our knowledge, this abdominal pigmentation does not seem to have any major adaptive function, and no cases of polyphenic control of abdominal pigmentation have yet been reported. Thus, it offers us an opportunity to investigate the mechanism underlying the phenotypic plasticity that has not yet been selected to become polyphenic. *O. fasciatus* is also amenable to RNA interference (Liu and Kaufman, 2009).

RNA interference

First discovered in the nematode worm, *Caenorhabditis elegans*, RNA interference (RNAi) is a natural response to the presence of double-stranded RNA (dsRNA) that results in sequence-specific gene silencing (Fire et al., 1998; Hannon, 2002). While RNAi had been observed using single-stranded RNA, it was Fire et al. (1998) who discovered that dsRNA was much more effective at interfering with normal gene expression. Exposure to dsRNA prompts the protein Dicer to slice the dsRNA into short double-stranded segments of ~20 nucleotides called siRNAs (Bernstein et al., 2001). One strand is then degraded and the other is incorporated into the RNA-induced silencing complex (RISC), a complex of endonucleases called argonaute proteins. This complex then degrades mRNA strands with sequences complementary to the siRNA sequence it contains, resulting in the loss of gene expression (Gregory et al., 2005; Leuschner et al., 2006). RNAi is now a widely used technique for genetic studies. dsRNA of the gene of study can be synthesized and

introduced to the organism through feeding or injection. RNAi is a highly effective technique for use in functional studies utilizing insects.

3. Project Aims and Goals

Although much is known about hormonal control of polyphenisms, how phenotypic plasticity of local pigmentation patterns is regulated remains unclear. One hypothesis is that hormonal control is inherent in phenotypic plasticity that has not yet been molded into a polyphenism through natural selection. Alternatively, it is possible that the hormonal control of a polyphenism is a property that evolves along with the evolution of a polyphenism. We think that the abdominal pigmentation of *O. fasciatus* offers a model to investigate the mechanisms underlying the beginning state of plasticity, prior to the evolution of polyphenisms. Thus, the goal of this study is to begin to investigate the genetic and hormonal control mechanisms underlying the phenotypic plasticity of *O. fasciatus* abdominal spot pigmentation and patterning. RNAi was used to knockdown EcR, FTZ-F1, and Met to study the hormone control mechanisms of abdominal pigmentation in *O. fasciatus*. Hox gene Abd-B was also knocked down to gain a better understanding of how patterning of milkweed bug abdominal spots are regulated.

MATERIALS AND METHODS

General Methods:

Animals

Milkweed bugs, *Oncopeltus fasciatus*, were obtained from the Carolina Biological Supply. They were raised at room temperature (26.5°C) on organic sunflower seeds and water in plastic containers with a photoperiod of 16 hours light, 8 hours dark. They were provided with cotton to lay their eggs on. For *Abd-B* RNAi experiments, milkweed bugs in treatment groups were also raised at 20°C and 33°C.

Imaging and quantification of pigmentation

Whole bodies of *O. fasciatus* at the desired stage were fixed with 3.7% formaldehyde for ten minutes before being rinsed in PBS and stored in 80% glycerol (20% PBS) at -20°C for up to one week. The ventral abdomen of each bug was excised from the animal and mounted on glass slides in 80% glycerol. Ventral abdomens were imaged using Nikon SMZ 1500 Microscope with 18.2 Color Mosaic diagnostic instruments Insight Firewire Spot 2megasample camera at 10X magnification. The area of melanic pigmentation measured using ImageJ program (developed at the U.S. National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/). The area was normalized to body size by dividing the area of the abdominal spots by the total area of 4 abdominal segments (A2-A5). All statistical analyses were performed using JMP Pro 9.

RNA interference (RNAi):

Ventral abdominal mRNA isolation and cDNA synthesis

Fourth and fifth instar *O. fasciatus* nymphs were dissected in 1X phosphate-buffered saline (PBS, 0.02M phosphate, 0.15M NaCl, 0.0028M NaH_2PO4, 0.0162M Na_2HPO4; pH 7.4). Gut and body fat was removed and remaining bodies were homogenized in Trizol (Invitrogen, www.invitrogen.com) using a RNAase-free polypropylene pellet pestle. Chloroform was added to the Trizol mixture in a ratio of 1:5 chloroform: Trizol mixture, and centrifuged at 14046 rcf at 4°C for 15 min. The supernatant containing RNA was extracted and transferred to another sterile microcentrifuge tube. Isopropanol was added at a 1:1 ratio before centrifugation at 14046 rcf at 4°C for 10 min. Resulting pellet was washed with 75% ethanol in diethylpyrocarbonate (DEPC)-treated MilliQ water (0.0001% DEPC in distilled water) and centrifuged at 5816 rcf at 4°C for 5 minutes. Pellet was re-suspended in 13 µl of DEPC MilliQ water and stored at -80°C. Isolated RNA was treated with DNAse (Promega) following the directions provided by the manufacturer and precipitated in isopropanol at a 1:1 ratio with an added 5% volume of 3M sodium acetate (pH 5.2). cDNA was synthesized from 1 µg of RNA using the cDNA synthesis kit following the manufacturer's instructions.

Polymerase chain reaction for the amplification of *EcR*, *FTZ-F1*, *Met,* and *Abd-B* and analysis by electrophoresis

The sequences for EcR, FTZ-F1, Met, and Abd-B were obtained from Ewen-Campen et al. (2011) and Angelini et al. (2005). Primers were designed using the Primer3 program, v.0.4.0 (http://frodo.wi.mit.edu) (Table 1). Polymerase chain reaction (PCR) mixture that contained 2% cDNA, PCR Buffer (1mM Tris, 5mM KCl, 0.15mM $MgCl_2$, pH 8.3), 0.2mM dNTPs, 0.4µM forward (FW) primer (Table 1) for each of the genes, 0.4µM reverse (RV) primer of each of the genes (Table 1) and 0.1units/µL Taq DNA Polymerase was prepared

in distilled water. The PCR program was as follows: for step 1 reaction was held at 94°C for 2 minutes once. Steps 2-4 reaction subsequently was held at 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute, and repeated for a total of 44 cycles. For Step 5, the reaction was incubated at 72°C for 5 minutes before ending at 4°C. Gel electrophoresis for 20 minutes at 100V was used to determine the success of each gene amplification and to isolate the PCR product.

Cloning reaction, transformation and linearization of plasmid DNA

Amplified cDNA product was isolated from the 1% agarose gel using the MinElute Gel Extraction Kit (Qiagen) following the manufacturer's instructions. Extracted cDNA was cloned into a TOPO® TA vector (Invitrogen) and added to TOP10 Chemically Competent bacterial cells (Invitrogen) at a ratio of 1:25 and incubated on ice for 20 minutes before heat shock for 30 seconds at 42°C. Super optimal broth with catabilite repression (SOC) medium (2% Bacto Tryptone, 0.5% yeast extract, 0.01M NaCl, 0.0025M KCl, 0.01M $MgCl_2$, 0.01 $MgSO_4$, 0.02M glucose) was added at a 5:1 ratio to the mixture. The transformation was completed by shaking the tube at 37°C for 1 hr at 250 rpm. The transformed cells were applied to prewarmed agar solid growth medium (1% Tryptone, 0.5% Yeast Extract, 1% NaCl, 2% agar) containing 0.1 mg/ml ampicillin, the surface of which was also treated with 30μL of ampicillin, and then incubated at 37°C overnight. Single colonies were picked and cultured at 37°C overnight in Luria broth (1% tryptone, 0.5% yeast extract, 1% NaCl) containing 0.1mg/ml ampicillin. Bacterial plasmid purification was carried out using the QIAprep Spin Miniprep Kit (Qiagen) following manufacturer's instructions. Plasmids were sequenced to confirm their identities. Plasmid DNA was linearized via restriction enzyme digestion.

Double-stranded RNA (dsRNA) synthesis and injection

dsRNA strands for each gene were synthesized using the T3 and T7 MEGAscript (Ambion) kits following manufacturer provided instructions. Equal amounts of single-stranded RNAs (ssRNAs) were hybridized to form 2 µg/µL solution of double-stranded RNA (dsRNA) in DEPC water. dsRNA was annealed in a thermal cycler using the protocol by Hughes and Kaufman (2000). For the first step, the mixture was maintained at 85°C for 3 minutes followed by a 20 minute ramp down to 55°C. For the second step, the mixture was maintained at 55°C for 10 min, followed by a 10 min ramp down to 40°C which was held for 20 min. The mixture then was subjected to a 5 min ramp down to 30°C where it was held for 10 min and then cooled to 4°C. The successful annealing of dsRNA product was confirmed using agarose gel electrophoresis.

All *O. fasciatus* Day-0 (D0) 4th instar and D0 5th instar nymphs were anesthetized on ice and injected through their dorsal abdominal segment. D0 4th instar nymphs were injected with 0.5 µg of dsRNA while D0 5th instar nymphs were injected with 1 µg of dsRNA of the respective gene using a 10-µL glass capillary needle connected to a syringe. As a control, 1 µg of *bacterial ampicillin-resistance* (*amp^{r}*) dsRNA. RNAi bugs were maintained at 26.5°C in plastic containers with sunflower seeds and maintained until death in order to characterize the phenotypes of treated animals. Dates of molting, major phenotypic changes observed, and death were recorded for each individual. Pictures were taken of animals at 10x magnification in 95% ethanol.

RNAi knockdown verification

Semi-quantitative Reverse transcriptase PCR and knockdown verification

Semi-quantitative reverse transcriptase PCR (RT-PCR) was done to confirm the knockdown of genes in dsRNA injected animals. *Ribosomal protein subunit 3* (*rps3*) was used as control for loading. D0 5th instar *O. fasciatus* nymphs were injected with 0.5 µg of dsRNA for *amp^{r}*, *Met, EcR,* or *FTZ-F1* dsRNA, and raised in normal conditions. After four days, RNA was isolated from these animals and converted to cDNA as described above (Materials and Methods: mRNA isolation and cDNA synthesis).

Relative expression profiling via RT-PCR

Forward and backward primers for all genes and for *ribosomal protein subunit 3* (*rps3*) as a loading control were used in RT-PCR using the same protocol and program as described above. The cycle numbers used were as follows: *rps3* = 23 cycles; *EcR*, *FTZ-F1* and *Met* = 35 cycles. Knockdown of Abd-B was previously verified (A. Sharma, unpublished).

Table 1. Primer sequences used in this study.

Gene		dsRNA Preparation Primer sequence
EcR		FW: 5’ GAGCCAAACCAGTCTCACC 3’
		RV: 5’ TGTCAAAGCCAGGTAATCGT 3’
FTZ-F1		FW: 5’ GAGATGACCCTCCCAAATG 3’
		RV: 5’AGAAGGAACTTTACGCAGATGTAG 3’
Met		FW: 5’ TAFCATTTGGAGTGCCTTCT 3’
		RV: 5’ CGGATTTCTCATTTGTGTGG 3’
Abd-B		FW: 5’GCCAACAACAACAACAGCA3’
		RV: 5’GGTGTTTCATGGCTCCAC3’
Amp^r		FW:
		RV:
Gene	# of cycles used	Knockdown verification primer sequence
EcR	35	FW: 5’ GCCACTTCTACTTTAGTTTGTACTC
		RV: 5’ CCATCCTCAACATCATTACCTC 3’
FTZ-F1	35	FW: 5’ ATGGTCGGATATGCTGGTT 3’
		RV: 5’ CTCGCCTTTTCATCTACTCCT 3’
Met	35	FW: 5’ GGTTTGCCACAATAGGAGAA 3’
		RV: 5’ ATCACTCACCCTGAAGACCA 3’
Rps3	30	FW: 5’ TTGATACCCAAAACCCCTTG 3’
		RV: 5’ CAACCCCATACACTTGACCT 3’

RESULTS

We sought to identify the hormonal and genetic mechanisms underlying the regulation of melanization and temperature regulated plasticity of the ventral abdominal spots of the milkweed bug, *Oncopeltus fasciatus*. We conducted a reverse genetics project to study the roles of Ecdysone receptor (EcR), Fushi tarazu-factor 1 (FTZ-F1), and Methoprene-tolerant (Met) in the hormonal control of melanization through post-transcriptional knockdown of these genes via RNA interference (RNAi). We knocked down the expression of these genes in Day 0 (D0) 4th and 5th instars and raised them at 26.5°C. We also knocked down the expression of Hox gene, *Abdominal-B* (*Abd-B*), in D0 5th instars and raised the treated animals at temperatures of 20°C, 26.5°C, and 33°C in order to investigate how genetic mechanisms regulate the melanization and plasticity of *O. fasciatus* abdominal patterning. Knockdown of all these genes, except *Abd-B* of which knockdown was previously verified (A. Sharma, unpublished), was confirmed in 26.5°C raised animals using semi-quantitative reverse transcriptase PCR (RT-PCR).

Knockdown verification shows successful knockdown of met, EcR, and *FTZ-F1*

Knockdown of these genes at 26.5°C was confirmed using semi-quantitative reverse transcriptase PCR (RT-PCR). When *met*, *EcR*, and *FTZ-F1* primers were used, the resulting PCR product synthesized from the cDNA generated from each of the respective knockdown animals showed that the gene was indeed knocked down through the presence of a comparatively less bright band compared to that produced by the other cDNA samples using the same primer (Fig. 2). We also did not observe *met* expression in *FTZ-F1* knockdown animals, indicating there is potentially an interaction between FTZ-F1 and Met

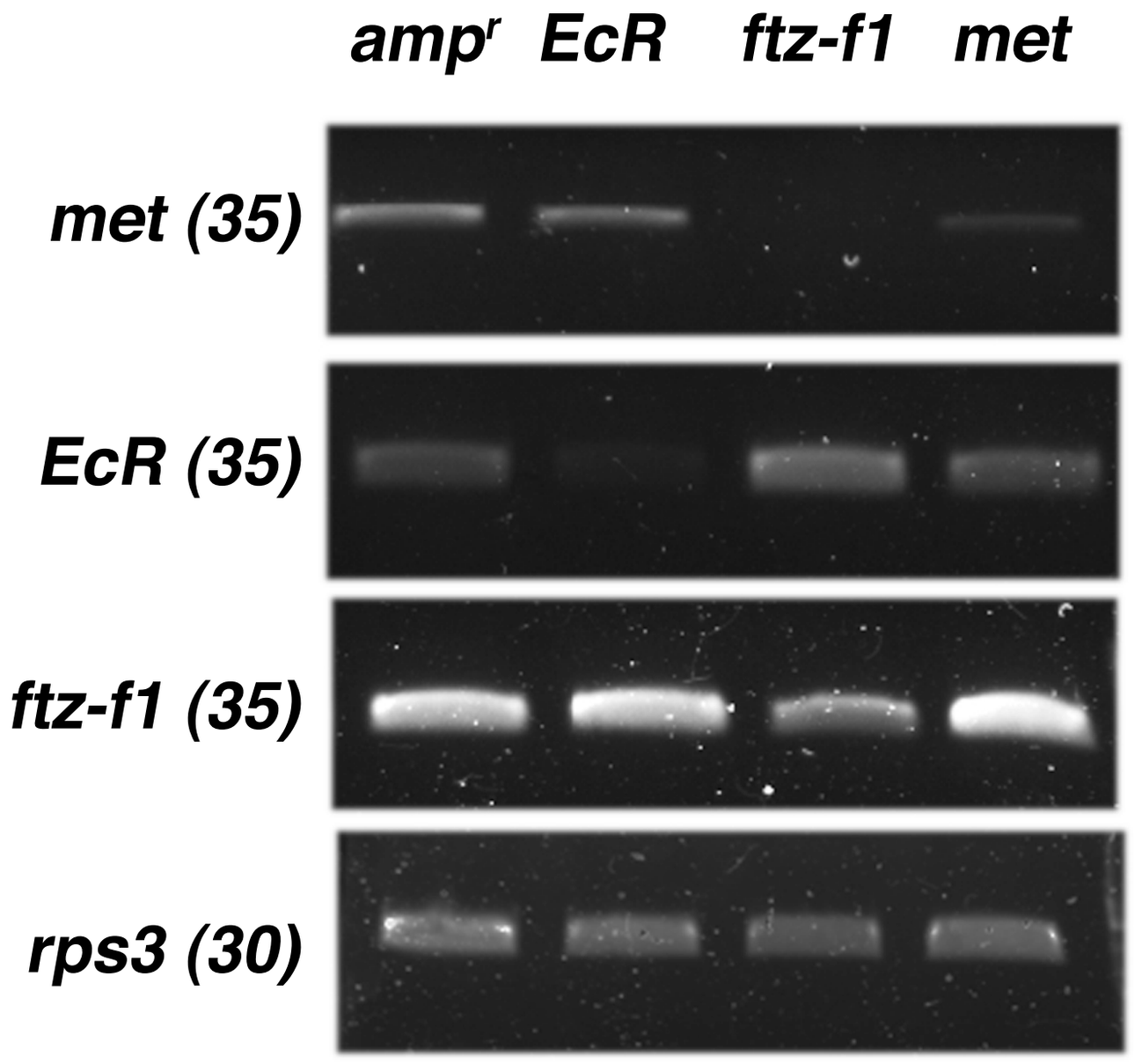


Figure 2. Knockdown verification for *EcR*, *FTZ-F1*, and *met* using semi-quantitative PCR. *Ribosomal protein subunit 3* (*rps3*) was used as a loading control while *amp*^{r} was used as a knockdown control. Expression of these genes was analyzed in D4 fifth animals that were treated with 0.5 µg of dsRNA of the respective genes as D0 5th animals. Animals were raised at 26.5°C. The numbers in brackets refer to the number of PCR cycles used for each gene. The image was taken with a Bio-rad Molecular Imager Gel Doc XR System.

expression (Fig. 2). *rps3* forward and reverse primers were used as a loading control and the bands showed RT-PCR and loading were successful (Fig. 2).

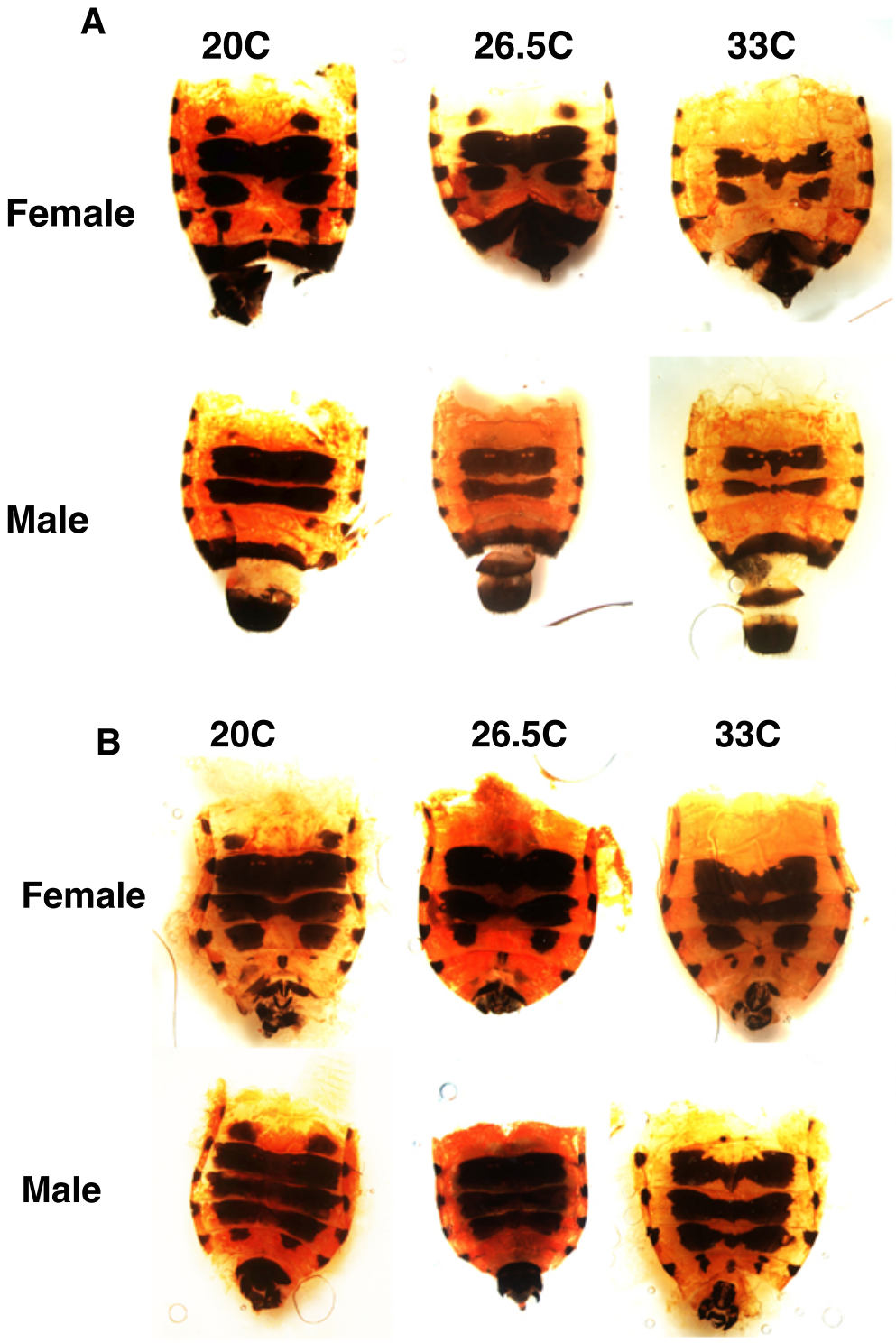
amp^r (control) animals exhibit phenotypic plasticity of ventral abdominal pigmentation

ampicillin resistance (*amp^r*) dsRNA injections on D0 4th and D0 5th *O. fasciatus* were used as a negative control for dsRNA injections. D0 4th and D0 5th animals received 0.5 µg (n = 14) and 1 µg (n=10), respectively of *amp^r* dsRNA. Treated D0 5th animals were also raised at 20°C and 33°C in addition to room temperature to determine the effects of temperature on control animals. D0 5th treated animals molted into adults an average 8.8 days after injection (Fig. 4A). D0 4th animals molted into 5th instars on average 6.1 days after injection and into adults on average 8.7 days after injection (Fig. 4B). *amp^r*-dsRNA treated males raised at 20°C (n=7), 26.5°C (n=6) and 33°C (n=5) had abdominal melanization that was significantly different between temperatures (ANOVA, F = 53.5, df = 2,15, p = 0.0001; Tukey HSD test; Figure 3). *amp^r* dsRNA-treated females raised at 20°C (n=3), 26.5°C (n=4) and 33°C (n=5) also had abdominal melanization that was significantly different between temperatures (ANOVA, F = 63.0, df = 2,9, p = 0.0001; Tukey HSD test; Figure 3).

I. Hormonal regulation of abdominal melanization

EcR knockdown inhibits molting

We injected D0 4th instars and D0 5th instars with 0.5 µg and 1 µg, respectively, of *EcR* dsRNA and maintained the animals at 26.5°C. We found that none of the Day 0 4th or 5th instar *EcR* dsRNA-injected animals were able to molt to the next instar (Fig. 5B, 5C).



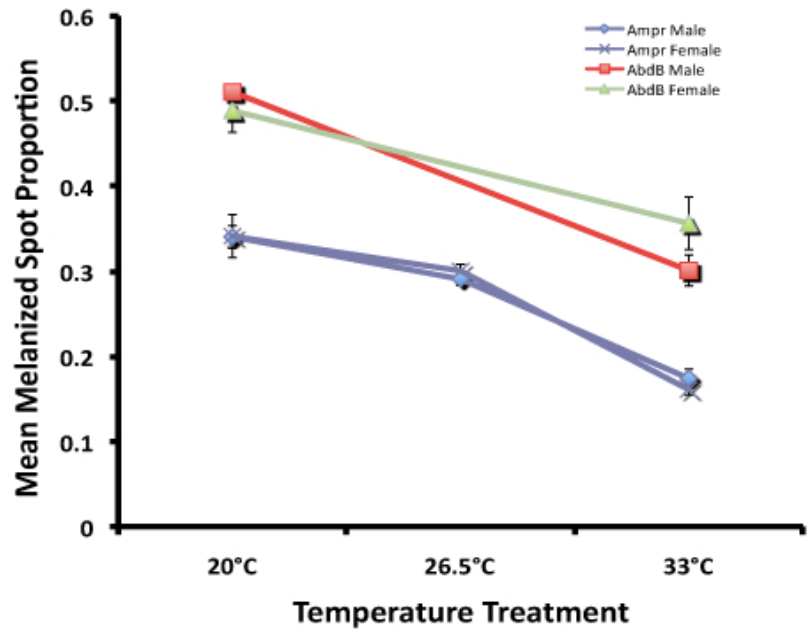
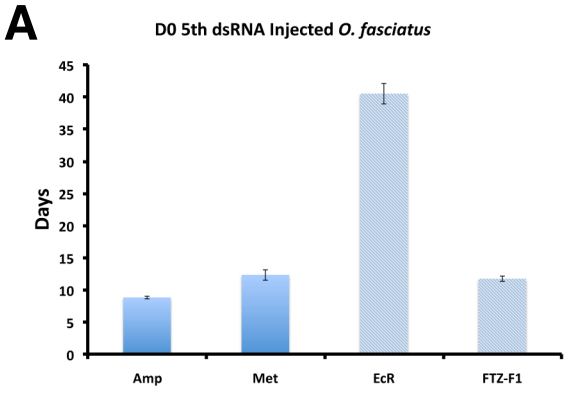


Figure 3. Effect of *amp*^{r} and *Abd-B* knockdown on the melanization and plasticity of ventral abdominal pigmentation in adult *O. fasciatus* at three different temperatures. D0 5th instars were injected with 0.5 µg dsRNA of either *amp*^{r} or *Abd-B* and raised at 20°C, 26.5°C, or 33°C. *amp*^{r} knockdown was used as a knockdown control. The ventral abdomen of each bug was mounted in 80% glycerol and imaged using Nikon SMZ 1500 Microscope with 18.2 Color Mosaic Diagnostic instruments Insight Firewire Spot 2megasample camera at 10X magnification. The proportion of the area of the melanized ventral abdominal spot normalized to the total abdominal area was calculated. A) *amp*^{r} knockdown animals raised at the three temperatures; Males (n=7, n=6 and n=5 for 20°C, 26.5°C, or 33°C, respectively): ANOVA, F = 53.5, df = 2,15, p = 0.0001; Tukey HSD test and Females(n=3, n=4 and n=5 for 20°C, 26.5°C and 33°C, respectively): ANOVA, F = 63.0, df = 2,9, p = 0.0001; Tukey HSD test, B) *Abd-B* knockdown animals raised at the three temperatures. Between 20°C and 33°C animals, Males (n = 3 and n = 3 for 20°C and 33°C, respectively): (ANOVA, F = 108.73, df = 1,4, p = 0.0005), Females (n = 4 and n = 6 for 20°C and 33°C, respectively): (ANOVA, F = 9.2422, df = 1,8, p = 0.0161), and C) comparison of the areas of *amp*^{r} and *Abd-B* knockdown female and male animals raised at the different temperatures.



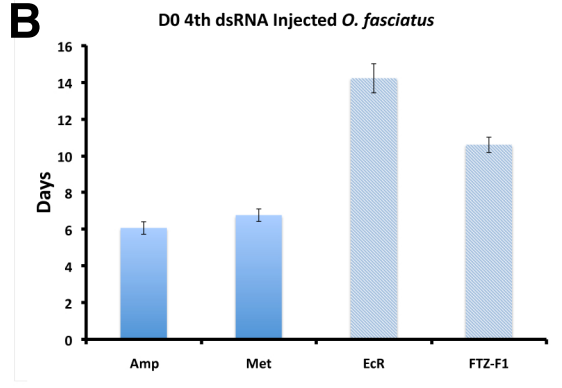


Figure 4. Phenotypic effects of *amp*^{r} and *met* RNAi on molting and *EcR* and *FTZ-F1* RNAi on *O. fasciatus* survival at 27°C. D0 4th and 5th animals were injected with 0.25 µg and 0.5 µg, respectively, of dsRNA. The mean number of days to molt after injection for *amp*^{r} and *met* dsRNA-treated animals and the mean days until death after injection for *EcR* and *FTZ-F1* dsRNA-treated animals were recorded. Sample sizes as reported in the text. Error bars made using SEM. A) D0 5th dsRNA-injected animals, B) D0 4th dsRNA-injected animals.

Animals treated as the D0 4th instar survived on average 14.2 days whereas D0 5th instars survived on average 40.2 days post injection (Fig. 4A, 4B).

EcR is required for adult abdominl melanization but not thoracic melanization

D0 4th instar injected animals (n = 9) displayed no unusual melanization expression even twenty days after injection (Fig. 5B). In contrast, D0 5th instar (n = 12) thoraces began to darken starting around nine days after injection and ultimately became heavily melanized as seen in the adult thoraces (Fig. 5C, 5D). Thus, the dorsal thorax between the wing pads, which is normally orange in the 5th instar, became melanized, and the ventral thorax also became melanized. However, the D0 5th injected animals expressed very little abdominal melanization, indicating that while adult thoracic pigment deposition was unaffected, the abdominal pigmentation was suppressed. All injected animals also developed a melanized black spot at the site of injection, which was not observed in *amp*^{r} dsRNA-injected D0 4th or D05th instars that molted into adults (Fig. 5A, 5D, 6A).

Ftz-f1 dsRNA-injected nymphs are unable to molt

We injected D0 4th instars (n = 25) and D0 5th instars (n = 15) with 0.5 μg and 1 μg, respectively, of *FTZ-F1* dsRNA and maintained the animals at 26.5°C. All *FTZ-F1* dsRNA-injected animals also did not molt to the next instar from either the 4th or the 5th instar and died on average 10.6 and 11.7 days post-injection, respectively (Fig. 4A, 4B). The D0 4th and D0 5th treated animals neither showed any signs of thoracic pigmentation nor developed abdominal pigmentation (Fig. 6F, 6G). All treated animals developed the melanized spot at the site of injection (Fig. 6F, 6G). Thus, while *FTZ-F1* dsRNA-injected nymphs, like EcR

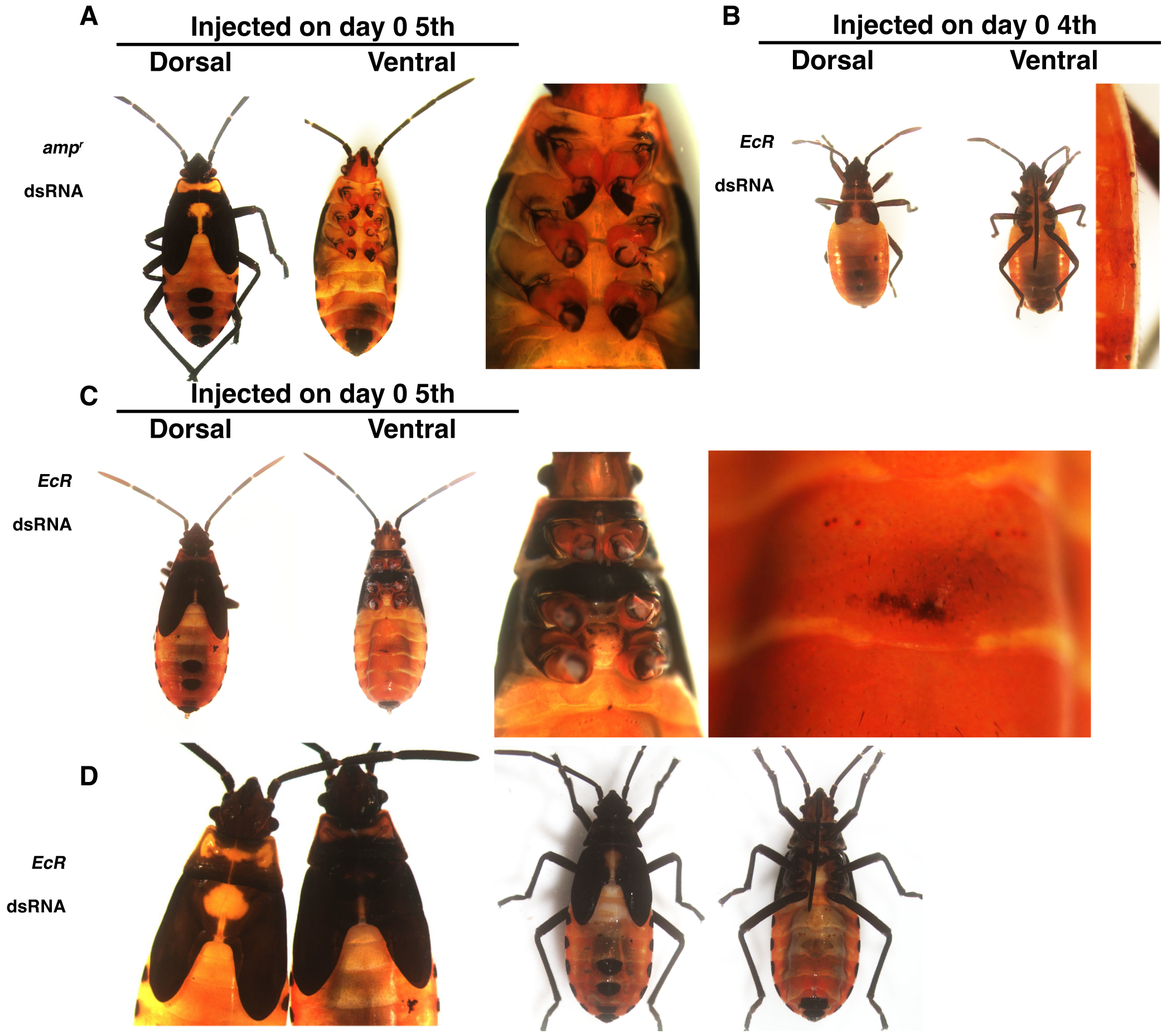


Figure 5. Phenotypic effects of *EcR* RNAi treatment on D0 4th and D0 5th instar *O. fasciatus*. Animals were fixed in 3.7% formaldehyde, stored in 80% glycerol, and imaged using Nikon SMZ 1500 Microscope with 18.2 Color Mosaic Diagnostic instruments Insight Firewire Spot 2megasample camera at 10X and 30x magnification. A) *amp*^{r} knockdown animal as a control. B) Dorsal and ventral images of D0 4th injected animals. C) D0 5th animals showing the pigmented thorax and lack of ventral abdominal pigmentation. D) Comparison of the pigmentation of the pronotum in animals injected with *amp*^{r} and EcR dsRNA as D0 5th instars.

dsRNA injected nymphs, are unable to molt, *FTZ-F1* dsRNA-injected nymphs have a shorter survival timeline and do not live long enough to develop adult melanization.

Methoprene-tolerant (Met) knockdown prevents molt to the adult stage and whole body melanization in a few adults

We injected *methoprene-tolerant* (*met*) dsRNA into D0 4th (n = 17) and D0 5th instars (n = 16) in order to investigate the effects of the loss of JH sensitivity on melanization. All *met* dsRNA-injected D0 5th instars and D0 4th instars molted into phenotypically normal adults and fifth instars respectively. It took on average 6.8 days for D0 4th instars to molt, and on average 12.3 days for D0 5th instars to molt (Fig. 4A, 4B). The *met* dsRNA-injected D0 5th instars took an average 3.5 days longer to molt to the normal adult stage than *amp^{r}* dsRNA-treated D0 5th instars (Fig. 4A). *met* dsRNA-injected Day 0 4th instars either molted into fifth instars after six days and became normal adults, or molted into fifth instars seven days or more after injection and then died as fifth instars. The D0 4th injected *O. fasciatus* that died as fifth instars did not develop thoracic or abdominal pigmentation (Fig. 6B).

There were occasional appearance of unusual morphology and melanization in *met* dsRNA injected D0 4th animals. The atypical morphology was characterized by splayed wings and short bodies with rounded abdomens (Fig. 6D). The bright orange coloration of a new molt these animals displayed did not deepen to a darker orange within a few hours as typically seen in other newly molted *O. fasciatus*, and animals remained a lighter orange at the pronotum even a few days after molting (Fig. 6D, 6E). The wings in *Oncopeltus* are characterized by two black marks at the distal end of the wing. The marks typically become melanized within a few hours after molting. However, in a few *met* dsRNA-injected animals, the distal mark remained white with traces of melanin being deposited at the wing margin

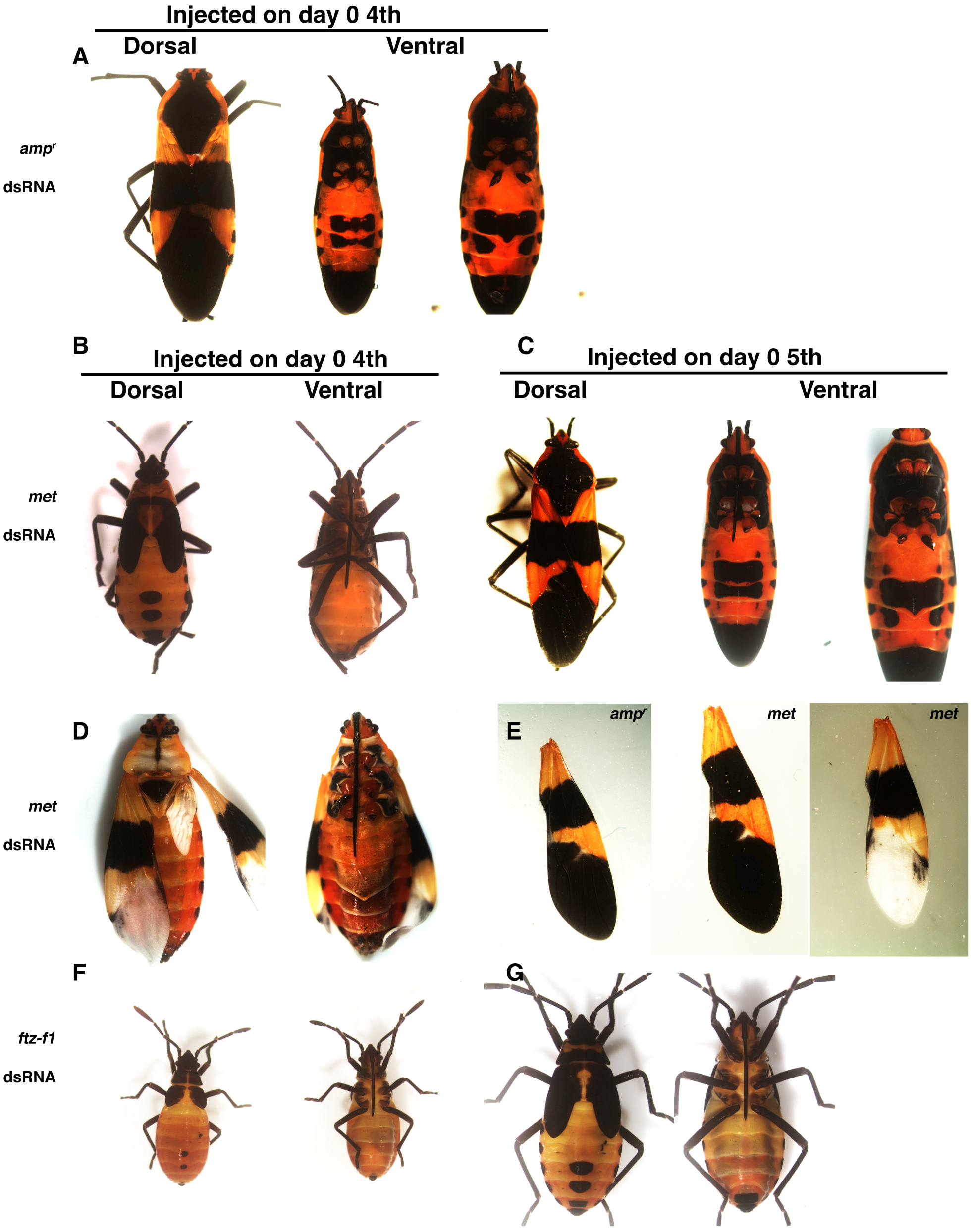


Figure 6. Phenotypic effects of *met* and *FTZ-F1* knockdown in D0 4th and D0 5th *O. fasciatus*. A) D0 4th *ampr* knockdown animals as a control. B) D0 4th *met* knockdown animals that molt to the 5th instar and fail to molt to adults do not display unusual phenotypes. C) D0 5th *met* knockdown animals both male and female do not display unusual phenotypes. D) Example of an unusual phenotype arising from D0 4th *met* knockdown animals. E) Wing melanization comparison between *ampr* and *met* knockdown animals, including the instance of unusual melanization. F) *FTZ-F1* knockdown animal treated with dsRNA as a D0 4th instar. G) *FTZ-F1* knockdown animal treated as a D0 5th instar. Animals were treated with 0.25 µg and 0.5 µg, respectively, of dsRNA.

even after a few days (Fig. 6E). There was some degree of thoracic melanization but it was mostly absent in these animals (Fig. 6D). These unusual animals also had limited ventral abdominal patterning and only expressed marks around the spiracles (Fig. 6D). These data suggest that JH plays a role in regulating the proper deposition of melanin at certain locations of the *O. fasciatus* adult body.

II. Hox regulation of abdominal melanization pigmentation patterning

Abd-B knockdown increases melanization

Animals treated with *Abd-B* dsRNA exhibited overall increased ventral abdominal melanization compared to wildtype or amp^r dsRNA-treated animals. In *Abd-B* dsRNA-treated females raised at 26.5°C, the A2 band exhibited a full stripe of melanization compared to the small spots or no melanization exhibited in the same band in wildtype female *O. fasciatus* (Fig. 3B). In *Abd-B* dsRNA-treated males raised at 26.5°C, there was the expression of a solid band of pigment in the middle of the A5 segment that was not seen in wildtype males raised at the same temperature (Fig. 3B). For all *Abd-B* dsRNA-treated animals, the melanization in the A6 abdominal segment which is seen in wildtype animals is not expressed while there is an increase in the melanization of other abdominal segments (Fig. 3B). This suggests that Abd-B plays a role in regulating either the synthesis or deposition of extra melanin in the ventral abdomen of *O. fasciatus* except for the second to last abdominal segment.

Abd-B does not affect temperature dependent ventral abdominal spot plasticity

Between the 20°C, 26.5°C, and 33°C raised *Abd-B* dsRNA injected animals of both sexes, there is evidence of temperature regulated phenotypic plasticity. In both males and females brought up at 33°C, the bands of melanization in each abdominal segment were not as broad as those of animals raised at both 26.5°C and 20°C (Fig. 3B). The area of abdominal pigmentation in *Abd-B* dsRNA-treated males raised at 20°C (n = 3) was significantly larger than that of *Abd-B* knockdown males raised at 33°C (n = 3) (ANOVA, F = 108.73, df = 1,4, p = 0.0005; Figure 3). The area of abdominal pigmentation in 20°C raised *Abd-B* dsRNA-treated females (n = 4) was also significantly larger than that of 33°C raised females (n = 6) (ANOVA, F = 9.2422, df = 1,8, p = 0.0161; Figure 3). The directionality of the reaction norm was not altered between Amp^r and *Abd-B* knockdown animals, implying that temperature regulated plasticity has not been altered in *Abd-B* knockdown animals (Fig. 3A, 3B). Taken together, our results suggest that *Abd-B* either plays a role in the synthesis or deposition of extra melanin within the animals, but does not play a role in regulating the plasticity of these ventral abdominal spots.

Discussion

Phenotypic plasticity is the expression of different phenotypes by individuals in a population with similar genotypes in response to different environments. All traits are inherently plastic to some degree as gene expression always occurs in context of the environment (Nijhout, 1994; Riddiford et al., 2000). A specific type of phenotypic plasticity is called a polyphenism, where a discrete switch exists in the phenotypes occurs along a continuous range of an environmental variable. Polyphenisms are largely regulated by developmental hormones (Nijhout, 1999; Nijhout, 2003). There are two possibilities for the evolution of hormonal control in polyphenisms: the hormonal control might be inherent in phenotypic plasticity and simply needs to be shaped by evolutionary forces, or the hormonal control of the polyphenism evolves *de novo* along with the evolution of the polyphenism through the co-option of hormonally mediated switches. In order to investigate the genetic and hormonal mechanisms underlying the regulation of *Oncopeltus fasciatus* abdominal spot melanization and plasticity, we conducted gene knockdowns of Ecdysone receptor (EcR), Fushi tarazu-factor 1 (FTZ-F1), Methoprene tolerant (Met) and the Hox gene, Abdominal-B (Abd-B).

I. Hormonal regulation of pigmentation and plasticity

a. *EcR and FTZ-F1 knockdown animals were unable to molt*

In our present study, we found that all *EcR* and *FTZ-F1* knockdown D0 4th and D0 5th animals were unable to molt into the next instar or the adult stage, respectively (Fig. 5B, 5C, 6F, 6G). EcR is a receptor in the ecdysone pathway that has been shown to play a role in molting among other developmental processes (Brown and Truman, 2009). In *M.*

sexta, ecdysone works through EcR and Ultraspiracle to initiate and orchestrate the molt (Hiruma and Riddiford, 2009). FTZ-F1 is an orphan nuclear receptor that has been shown to work downstream of ecdysone in the 20E signaling pathway in *M. sexta* (Riddiford et al., 1999; Hiruma and Riddiford, 2001). In the hemimetabolous insect, the german cockroach *Blatella germanica*, FTZ-F1 has been shown to be necessary for successful completion of ecdysis (Cruz et al., 2008) and found to be required for the maturation of cuticular denticles (Ruaud et al., 2010). Our findings are consistent with these previous studies implicating FTZ-F1 as a part of the endocrine regulation underlying ecdysis. The inability to molt suggests that EcR and FTZ-F1 knockdown in *O. fasciatus* successfully interfered with the ecdysone signaling cascade to a certain degree, indicating that the hormonal cascade underlying molting are likely conserved across insects.

b. *EcR suppresses abdominal pigmentation but not thoracic pigmentation*

We found that D0 5th *EcR* dsRNA-injected animals developed thoracic pigmentation and abnormal ventral abdominal pigmentation on average after nine days post-injection (Fig. 5C). The loss of pigmentation is similar to the effects of knocking down EcR in the rice planthopper, *Laodelphgax striatellus,* where its knockdown leads to the loss of melanization in the wings (Wu et al., 2012). The development of thoracic but not ventral abdominal melanization in *O. fasciatus* suggests that *EcR* knockdown suppresses the melanin production or deposition only in the ventral abdomen. We know from previous temperature-mediated plasticity studies in the lab that only abdominal pigmentation is plastic and that the sensitive period for the plasticity is in the fifth instar (A. Sharma, unpublished). Given that only the abdomen, but not the thorax, is phenotypically plastic,

the results suggest an intriguing possibility that EcR only regulates the melanization of phenotypically plastic patterning in *O. fasciatus*. In other words, phenotypically plastic melanin patterning and non-plastic melanin patterning appear to be regulated by separate control mechanisms.

Our data suggest that the hormonal control mechanisms may already be in place to influence phenotypic plasticity in pigmentation, and just have to be shaped by natural selection for the evolution of the polyphenism. Future studies should focus on further exploring the extent to which *O. fasciatus* abdominal melanization is under endocrinal control. Gaining a better understanding of how the patterning of melanization is controlled could help unravel how this plasticity is regulated in these bugs. This could be undertaken through the application of ecdysteroid analogs to the ventral abdomens of D0 5th animals, and observing whether it affects the development of melanization on the ventral abdominal when animals molt into adults. Future studies should also investigate whether *O. fasciatus* abdominal spot plasticity can be selectively bred into a polyphenism and how the endocrine regulation of the plastic trait evolves.

c. *FTZ-F1 animals show no atypical pigmentation*

We found that D0 4th and 5th *FTZ-F1* dsRNA-injected animals, while failing to molt, otherwise appeared phenotypically normal with no development of pigmentation (Fig. 6F, 6G). This is potentially due to the fact that all animals died within 12 days after injection and did not have the time to develop such unusual pigmentation (Fig. 4). Thus, at this point, we cannot infer whether or not FTZ-F1 plays a role in regulating melanization. It is an orphan nuclear receptor whose role has not been characterized previously in *O. fasciatus*.

d. *Met knockdown animals have limited ability to molt and do not develop unusual pigmentation*

We found that *met* dsRNA-injected D0 5th animals were able to molt into phenotypically normal adults, while most D0 4th injected animals were able to molt to fifth instars but were not able to molt into the adult stage (Fig. 6B, 6C). Some D0 4th injected animals were successfully able to molt into seemingly phenotypically normal adults, but this potentially resulted from an incomplete knockdown of Met expression. Higher doses of dsRNA should be tested in the future. The inability to molt into the adult stage by D0 4th dsRNA treated animals may be because either these nymphs may have precociously metamorphosed internally into adults due to their JH insensitivity, or because JH sensitivity is required for initiating a molt in *O. faciatus*. JH has been found to act as a *status quo* hormone and its removal leads to the progression to the next life stage (Riddiford, 1996). It has been found that *met* RNAi *Tribolium* also experience ecdysis defects (Konopova and Jindra, 2007). The delay in molting we found suggests that Met in *O. fasciatus* might also play a role in regulating the timing of molting, potentially by influencing the ecdysone pathway.

While in the majority of Met knockdown animals, there were no unusual effects on melanization, there were occasional instances of animals developing abnormal pigmentation and morphology (Fig. 6D, 6E). These animals had delayed pigmentation deposition in the ventral abdomen, the pronotum and the distal wing marks (Fig. 6D, 6E). The distal mark on the wing lacked melanin at the time of molting, and even over time, only minimal melanin deposition was observed (Fig. 6E). In these insects, the hind wing could

not expand, indicating some sort of defect in hemolymph circulation. Wing expansions require the pressure exerted by hemolymph from the abdomen. In *D. melanogaster*, it has been found that animals with deficits in their ability to expand their wings also melanize slowly over the course of days rather than hours (Luan et al., 2006). Thus, Met could potentially be involved in regulating wing development and melanization through proper hemolyph circulation.

The lack of melanization seen in the few D0 4th instar *met* dsRNA-treated animals that molted into the animal exhibiting the unusual phenotype suggests that *met* may be required for melanin deposition as adults and that JH is involved in the regulation of the deposition of melanin in certain parts of *O. fasciatus*. The normal ventral abdominal spot patterning in the D0 5th instar *met* dsRNA-treated animals that subsequently molted into adults suggests that JH sensitivity does not play a role in regulating the plasticity of the ventral abdominal melanization although the possibility of incomplete Met knockdown remains. This information is in contrast to *M. sexta* where JH inhibits melanin synthesis in the cuticle and JH removal leads to two-fold increases of DDC (Curtis et al., 1984; Hiruma and Riddiford, 2009). Thus, in these insects, JH inhibits melanin synthesis whereas in *O. fasciatus*, we did not observe any obvious roles of JH in melanin production.

e. <u>*No Met expression in FTZ-F1 knockdown animals*</u>

We found that during knockdown verification using *met* primers, cDNA synthesized from FTZ-F1 knockdown animals appeared not to express *met* (Fig. 6F, 6G). Previous studies in *D. melanogaster* show that JH activates E75A through an intracellular pathway involving FTZ-F1, which acts as a competence factor that facilitates gene expression

(Dubrovsky et al., 2011). Given that these two proteins interact to regulate target genes (Bernardo and Dubrovsky, 2012), it is possible that FTZ-F1 might be involved in activating Met. The relationship between FTZ-F1 knockdown and *met* expression in *O. fasciatus* would be interesting to study in greater detail, using more sensitive quantitative tools, such as quantitative real time PCR.

II. Hox gene Regulation of Pigmentation Patterning and Plasticity

a. Abd-B knockdown leads to increased pigment deposition

Abd-B knockdown animals of both sexes showed an increase in the area of melanization in the segments where there is normally no pigmentation in control adults (Fig. 3B). *Abd-B* dsRNA-treated females expressed an abdominal spot in the A2 segment which was not seen in amp^r dsRNA-treated females (Fig. 3A, 3B). In *Abd-B* dsRNA-treated males, an abdominal spot was expressed in the A5 segment that was not observed in untreated animals (Fig. 3B). Thus, these data suggest that Abd-B plays a role in suppressing the deposition of melanin in the ventral abdomen. Our findings are similar to what is observed when Abd-B expression is silenced in *O. fasciatus* embryos (Angelini et al., 2005). In these embryos, two small spots of ectopic melanization appear in the first instar nymph when Abd-B is silenced. The loss of pigmentation of the most posterior abdominal segment in both sexes after Abd-B knockdown suggests that the normal function of Abd-B is involved in promoting the deposition of melanin in that segment (Fig. 3B). Similarly, in *Abd-B* RNAi *O. fasciatus* embryos, pigmentation is missing in the A10 segment, the second to last abdominal segment of the embryo, when Abd-B expression was removed.

b. Abd-B does not affect temperature-regulated plasticity of the ventral abdomen

We found that *Abd-B* knockdown animals raised at 20°C and 33°C still exhibited signs of temperature-dependent plasticity (Fig. 3B). In both males and females, the mean pigmented area of the ventral abdomen of animals raised at 20°C was significantly larger than that of animals raised at 33°C. Our results suggest that while Abd-B plays a role in regulating the deposition of pigmentation in the ventral abdomen, it does not affect temperature-dependent plasticity of these spots. Thus, unlike in *D. melanogaster*, Abd-B appears not to play a role in regulating the plasticity of abdominal pigmentation (Gibert et al., 2007). We propose that in *O. fasciatus,* Abd-B is involved in the pre-patterning of the abdominal melaninization. In other words, Abd-B determines which segment melanin can be deposited whereas some other mechanism, possibly EcR, regulates the plasticity of the abdominal pigmentation.

III. The hormonal and genetic regulation of pigmentation

Based on our findings that EcR knockdown results in the development of pigmentation in the thorax but not in the ventral abdomen, and that Abd-B appears to repress the deposition of melanin in the ventral abdominal segments, we propose that within *O. fasciatus*, the ecdysone signaling pathway regulates ventral abdominal pigmentation plasticity while Abd-B regulates the positioning of melanization in abdominal segments (Fig. 7). How the ecdysone-signaling pathway regulates the temperature dependent changes in abdominal melanization remains to be explored in future studies. Ecdysone could potentially be involved in the regulation of the increased melanization at lower temperatures, and decreased melanization at higher temperatures. Abd-B expression appears to act as a pre-pattern regulator, potentially by influencing another portion of the

melanin synthesis pathway. Furthering our understanding of the hormonal and genetic mechanisms behind the regulation of temperature-induced plasticity of the ventral abdomen in future studies could provide insight into the evolution of a polyphenism and threshold traits in general.

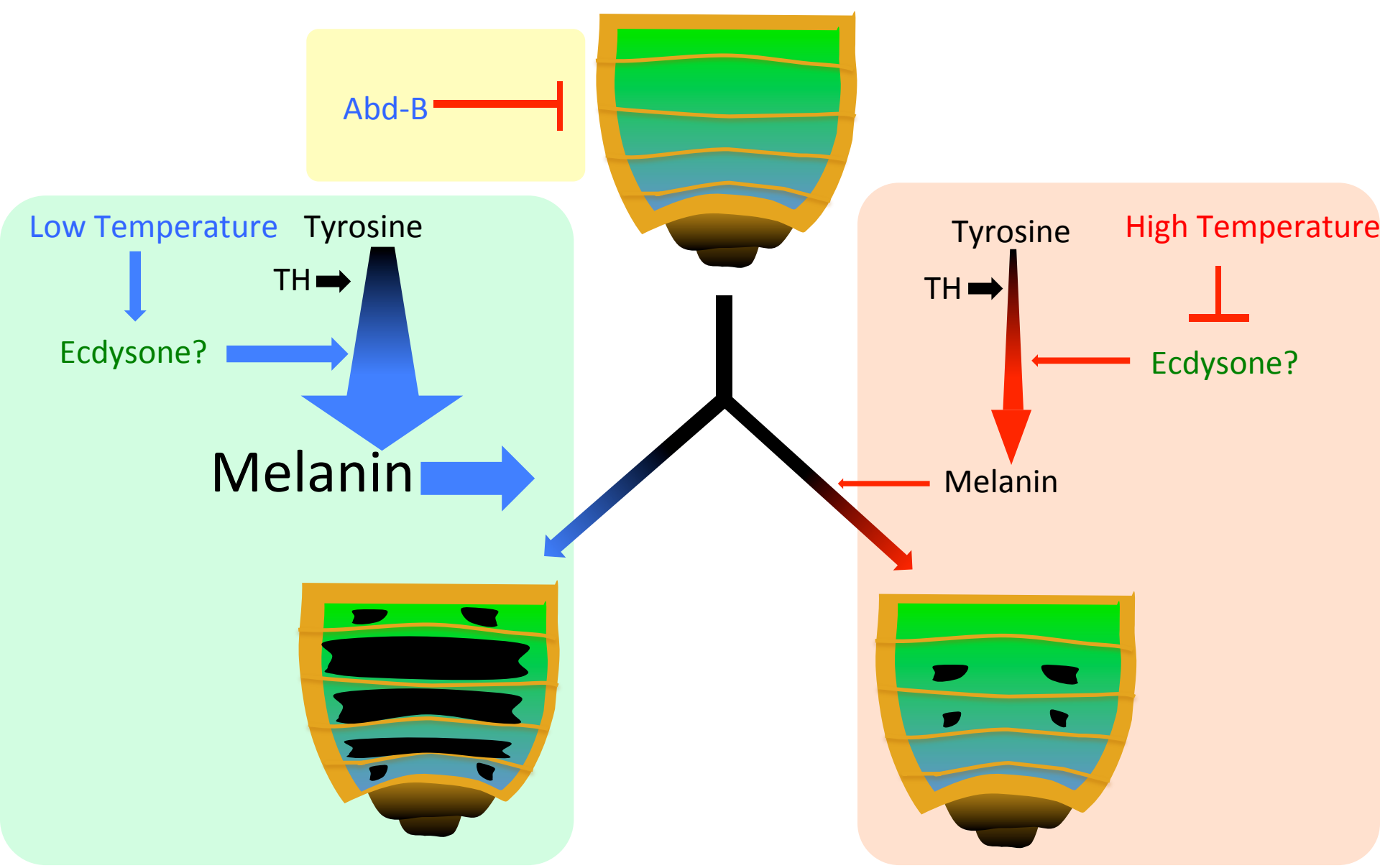


Figure 7. Proposed model for the regulation of ventral abdominal pigmentation in *O. fasciatus*.

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